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Request for grant of a patent

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1. Your reference	P32365/LMC/GST	
2. Patent application number *(The Patent Office will fill in this part)*	0228139.2	03 DEC 2002
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	Dr Olga Kozlova-Zwinderman 239/5 Gilmerton Road Edinburgh EH16 5TH	
Patents ADP number *(if you know it)*	08519084001	
If the applicant is a corporate body, give the country/state of its incorporation		
4. Title of the invention	"Fungal Biosensor for Contaminant Detection"	
5. Name of your agent *(if you have one)*	Murgitroyd & Company	
"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Scotland House 165-169 Scotland Street Glasgow G5 8PL	
Patents ADP number *(if you know it)*	1198013	

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application	Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
a) any applicant named in part 3 is not an inventor, or
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Claim *(s)*	-
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Priority documents	-
Translations of priority documents	-
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	-
Request for preliminary examination and search *(Patents Form 9/77)*	-
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Any other documents *(please specify)*	-

11. I/We request the grant of a patent on the basis of this application.

Signature Murgitroyd & Company
Murgitroyd & Company

Date
2 December 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

Gordon Stark 0141 307 8400

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"Fungal Biosensor"

The present invention provides a method of using fungus for determining the presence of at least one toxic substance in a sample and for assisting in the identification of the toxicant(s). More specifically there is provided a toxicity assay for use in determining the presence of toxins and in particular heavy metals and organophenols.

The release of contaminating substances into an environment such as a waterway or an area of agricultural land can have serious effects on the ecosystems found in that environment. It is important to be able to analyse these effects both prior to the release of such contaminants so as to manage their treatment or release, and after release so as to determine and counteract their effects.

Current methods used to monitor water quality and screen effluent generally involve chemical toxicity tests. However, these tests require a general idea

of the type of contaminant being tested for and can be very expensive.

Biosensors are also used for toxicity testing and are well known in the field. Toxicity depends on a variety of factors including pH, temperature, salinity and contaminant concentration, but depends especially on the test organism used in the sensor.

One of the most commonly used organisms is the bioluminescent bacterium, *Vibrio fischeri*. The bioluminescence involved is mediated by the luciferin-luciferase enzyme system wherein light emission is dependent on the electron transfer chain. Any disruption to the electron transfer chain, for example on exposure to a toxicant, affects light emission. Measurement of changes in light emission is therefore indicative of the presence of a toxic substance.

This system, however, only provides a simple indication of whether a contaminant is toxic or not. No detailed information is obtained on how toxic the contaminant is, nor is the contaminant identified.

The term toxicant as herein described relates to compounds which are toxic to fungus and also to any substance or compound with anti-fungal activity.

According to the present invention there is provided

an assay for use in determining the presence of a known toxicant in a test sample, the assay comprising the steps of;

- exposing a fungi transformed with a recombinant aequorin gene to a test sample of a substance,

- measuring the luminescence produced by the fungi,

- converting the luminescence data into a cytosolic free calcium ion concentration trace,

- and comparing at least one parameter of the cytosolic free calcium ion concentration trace with a bank of known toxicity reference data.

Preferably the cytosolic free calcium ion trace is a plot of the cytosolic free calcium ion concentration against time.

Preferably the fungi transformed with a recombinant aequorin gene is a filamentous fungi.

More preferably the fungi is of the *Aspergillus* species.

Most preferably the fungi is *Aspergillus awamori*.

Most preferably still the strain of *Aspergillus awamori* is strain 66A.

Preferably the substance is a contaminant.

Preferably the substance is a contaminated sample.

Preferably the parameter is at least one or more selected from the group comprising; lag time, rise time, amplitude, full width half maximum, number of cytosolic free calcium ion concentration increases, percentage increase in final cytosolic free calcium ion concentration resting level, and percentage increase in recovery time.

In a further embodiment of the invention the test sample is added in advance of the application of a stimulus to the test sample.

Preferably the stimulus is at least one or more from the group comprising; mechanical perturbation, hypo-osmotic shock, and change in external calcium chloride concentration.

Preferably the test sample is added 1 minute to 1 hour prior to the application of the stimulus.

More preferably the test sample is added 5 minutes prior to the application of the stimulus.

More preferably the test sample is added 30 minutes prior to the application of the stimulus.

In such instances, the parameters may include at least one or more selected from the group comprising; lag time, rise time, amplitude, full width half maximum, number of cytosolic free calcium ion concentration increase, percentage increase in final cytosolic free calcium ion concentration resting level, percentage increase in recovery time, and percentage increase in pre-stimulating cytosolic free calcium ion concentration resting level.

Preferably luminescence is measured for between 1 minute and 5 hours following the application of the stimulus.

More preferably luminescence is measured for 5 minutes following the application of the stimulus.

A further embodiment of the present invention provides a method of determining the amount of a contaminant present in a test sample comprising the steps of;

- exposing a strain of fungi transformed with a recombinant aequorin gene to a sample,

- measuring the luminescence produced by the fungi,

- converting the luminescence data into a cytosolic free calcium ion concentration trace,

- and comparing at least one parameter of the cytosolic free calcium ion concentration trace with a bank of pre-prepared toxicity reference data.

Preferably the cytosolic free calcium ion trace is a plot of the cytosolic free calcium ion concentration against time.

Preferably the fungi transformed with a recombinant aequorin gene is a filamentous fungi.

More preferably the fungi is of the *Aspergillus* species.

Most preferably the fungi is *Aspergillus awamori*.

Most preferably still the strain of *Aspergillus awamori* is strain 66A.

Preferably the substance is a contaminant.

Preferably the substance is a contaminated sample.

Preferably the parameter is at least one or more selected from the group comprising; lag time, rise time, amplitude, full width half maximum, number of cytosolic free calcium ion concentration increases, percentage increase in final cytosolic free calcium ion concentration resting level, and percentage increase in recovery time.

In a further embodiment of the invention the test sample is added in advance of the application of a stimulus.

Preferably the stimulus is at least one or more from the group comprising; mechanical perturbation, hypo-osmotic shock, and change in external calcium chloride concentration.

Preferably the test sample is added 1 minute to 1 hour prior to the application of the stimulus.

More preferably the test sample is added 5 minutes prior to the application of the stimulus.

More preferably the test sample is added 30 minutes prior to the application of the stimulus.

In such instances, the parameters may include at least one or more selected from the group comprising; lag time, rise time, amplitude, full width half maximum, number of cytosolic free calcium ion concentration increase, percentage increase in final cytosolic free calcium ion concentration resting level, percentage increase in recovery time, and percentage increase in pre-stimulating cytosolic free calcium ion concentration resting level.

Preferably luminescence is measured for between 1 minute and 5 hours following the application of the stimulus.

More preferably luminescence is measured for 5 minutes following the application of the stimulus.

A first experiment comprises testing the effect of pre-incubation of *Aspergillus awamori* with toxicants on cytosolic free calcium ion concentration response to an increase in external calcium chloride.

A further set of experiments described herein shows attempts to obtain characteristic data for a range of different toxicants at a number of different concentrations. The results demonstrate that each toxicant at each concentration produces a distinctive cytosolic free calcium ion concentration trace whose traits could be used to identify and characterise a toxicant present in a test sample.

A final experiment attempts to determine whether it is possible to identify and characterise individual toxicants from testing samples of mixtures of toxicants in different proportions. The traces produced are distinct for each mixture.

These results show that it is possible to characterise and identify a specific toxicant from a test sample by using the characteristic data obtained from a cytosolic free calcium ion concentration trace.

The parameters referred to herein relate to the following;

Lag Time, the time from addition of the test sample to the time when the cytosolic free calcium ion concentration, $[Ca^{2+}]_c$, began to rise;

Rise Time, the time from addition of the test sample to the time at which maximum $[Ca^{2+}]_c$ was reached;

Amplitude, the maximum $[Ca^{2+}]_c$ reached during the experiment;

Full Width Half Maximum, the width of the transient at the point where the amplitude equals half of the maximum amplitude of the transient;

Number of $[Ca^{2+}]_c$ Rises, the number of peaks in $[Ca^{2+}]_c$;

Percentage Increase in Final $[Ca^{2+}]_c$ Resting Level, the percentage increase in resting $[Ca^{2+}]_c$ at the end of the experiment, where the control value is taken to be 100%;

Percentage Increase in Recovery Time, percentage increase in recovery time where recovery time represents the total amount of $[Ca^{2+}]_c$ released during the period of time from the point when the maximum amplitude following calcium chloride treatment was achieved to the point when the $[Ca^{2+}]_c$ reached its final resting level. Recovery time was initially calculated for control cultures. In the control this period of time was calculated as 250

seconds. For the cultures subjected to the treatment with toxicant(s) the total amount of $[Ca^{2+}]_c$ was calculated for the same period of 250 seconds starting from the maximum amplitude. The recovery time of the control cultures was therefore:

$$\frac{\text{total amount of } [Ca^{2+}]_c \text{ (µM) for the toxicant-treated samples over 250 seconds} \times 100}{\text{total amount of } [Ca^{2+}]_c \text{ (µM) for the control sample over 250 seconds}}$$

; and

Percentage Increase in pre-Stimulating $[Ca^{2+}]_c$ Resting Level, the percentage increase in $[Ca^{2+}]_c$ prior to the stimulus, where the control value is taken to be 100%.

There is also the possibility of combining one or more of these parameters to obtain further values which can be used for identification of the toxicants in the mixture. For example, the summation of amplitude and recovery time will give the value of total cytosolic free calcium ions emitted from the time when $[Ca^{2+}]_c$ reaches its peak. Also summation of lag time and rise time will give the total time required for $[Ca^{2+}]_c$ to reach its peak. The division of final $[Ca^{2+}]_c$ resting level onto the pre-stimulation $[Ca^{2+}]_c$ resting level will show how many times the $[Ca^{2+}]_c$ resting level has changed after stimulation. Similarly, a division of the final $[Ca^{2+}]_c$ resting level onto the initial

$[Ca^{2+}]_c$ resting level prior to the addition of toxicant(s) gives further identifying data. Additionally, the summation of all the data points of the trace gives the total amount of cytosolic free calcium ions released during the monitoring period.

The present invention will now be described with reference to the following non-limiting examples and with reference to the figures, wherein:

Figure 1 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM external $CaCl_2$, following a 5 minute pre-incubation with different concentrations of 3,5-DCP.

Figure 2 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM external $CaCl_2$, following a 5 minute pre-incubation with different concentrations of Cr^{6+}.

Figure 3 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM external $CaCl_2$, following a 5 minute pre-incubation with different concentrations of Zn^{2+}.

Figure 4 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM external $CaCl_2$, following a 30 minute pre-incubation with different concentrations of 3,5-DCP.

Figure 5 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM external $CaCl_2$, following a 30 minute pre-incubation with different concentrations of Cr^{6+}.

Figure 6 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM external $CaCl_2$, following a 30 minute pre-incubation with different concentrations of Zn^{2+}.

Figure 7 shows the characteristic cytosolic free calcium ion concentration, $[Ca^{2+}]_c$, trace produced on addition of 5mM $CaCl_2$ following a 5 minute pre-incubation with different concentrations of 3,5-dichlorophenol, 3,5-DCP.

Figure 8 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM $CaCl_2$, following a 30 minute pre-incubation with different concentrations of 3,5-DCP.

Figure 9 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM $CaCl_2$, following a 5 minute pre-incubation with different concentrations of chromium ions, Cr^{6+}.

Figure 10 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM $CaCl_2$, following a 30 minute pre-incubation with different concentrations of chromium ions, Cr^{6+}.

Figure 11 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM $CaCl_2$, following a 5 minute pre-incubation with different concentrations of zinc ions, Zn^{2+}.

Figure 12 shows the characteristic $[Ca^{2+}]_c$ trace produced on addition of 5mM $CaCl_2$, following a 30 minute pre-incubation with different concentrations of zinc ions, Zn^{2+}.

Figure 13 shows the values of $[Ca^{2+}]_c$ trace parameters characteristic for different concentrations of pentochlorophenol, PCP; sodium dodecyl sulphate, SDS; and Toluene. Parameters assessed are Lag Time, LT; Rise Time, RT; Amplitude, A; Full Width Half Maximum, FWHM; Percentage Increase in pre-Stimulating $[Ca^{2+}]_c$ Resting Level, %IpreSRL; Percentage Increase in Final $[Ca^{2+}]_c$ Resting Level, %IFRL; Percentage Increase in Recovery Time, %IRT; and Number of $[Ca^{2+}]_c$ Increases.

Figure 14 shows the values of $[Ca^{2+}]_c$ trace parameters characteristic for 3,5-DCP, PCP, Zn^{2+}, Cr^{6+}, Toluene, and SDS. Parameters assessed are Lag Time, LT; Rise Time, RT; Amplitude, A; Full Width Half Maximum, FWHM; Percentage Increase in pre-Stimulating $[Ca^{2+}]_c$ Resting Level, %IpreSRL; Percentage Increase in Final $[Ca^{2+}]_c$ Resting Level, %IFRL; Percentage Increase in Recovery Time, %IRT; and Number of $[Ca^{2+}]_c$ Increases.

Figure 15 shows the values of $[Ca^{2+}]_c$ trace parameters characteristic for different mixtures of toxicants. Parameters assessed are Lag Time, LT; Rise Time, RT; Amplitude, A; Full Width Half Maximum, FWHM; Percentage Increase in pre-Stimulating $[Ca^{2+}]_c$ Resting Level, %IpreSRL; Percentage Increase in Final $[Ca^{2+}]_c$ Resting Level, %IFRL; Percentage Increase in Recovery Time, %IRT; and Number of $[Ca^{2+}]_c$ Increases.

Effect of pre-incubation of *Aspergillus awamori* with toxicants on $[Ca^{2+}]_c$ response to external calcium chloride

12 ml of sterile VS medium was inoculated with 1 x 10^5 spores per ml *A. awamori* strain 66A. 100 µl of the inoculated medium was added to each well of a 96-well plate and cultured in a humidity chamber in the presence of free water at 30 °C for 24 hours.

The following toxicants were tested: 3,5-dichlorophenol, zinc sulphate, and potassium dichromate. Each toxicant was added in a total volume of 25 µl VS medium or water 5 or 30 minutes before addition of 5 mM calcium chloride.

Luminescence was monitored for 5 minutes following addition of $CaCl_2$. Aequorin was completely discharged by adding 3M calcium chloride in 20% ethanol. The total concentration is thus 1.5 M calcium chloride in 10% ethanol.

Luminometry was performed using an EG & G Berthold (Bad Wildbad, Germany) LB96P Microlumat luminometer. Luminescence data was converted from real light units to $[Ca^{2+}]_c$ values using the following equation:

$$PCa = 0.332588 (-\log k) + 5.5593,$$

where k = luminescence counts per second/total luminescence counts. Total luminescence is measured as an integral of all luminescence up to complete aequorin discharge.

The following parameters were assessed:
Rise Time, Amplitude, Full Width Half Maximum and Final $[Ca^{2+}]_c$ Resting Level.

<u>Effects of different concentrations of toxicants on $[Ca^{2+}]_c$ traces</u>

Aspergillus awamori were transformed with an expression vector (pAEQ1-15) comprising a gene for synthetic apoaequorin (*aeqS*) under the control of the constitutive glucose-6-phosphate dehydrogenase promoter (*gpdA*).

These transformants were cultured in 100 µl of Vogel's medium with 1% sucrose (VS medium) in microwell plates for 24 hours before addition of a toxicant or a control of distilled water. Toxicants were dissolved in water to give the concentrations shown below. 25 µl of the each of the following concentrations were added to each culture:

TOXICANT	CONCENTRATIONS (mg/l)
3,5-dichlorophenol (3,5-DCP)	0.112, 11.2, 112
Chromium ions (Cr^{6+})	15, 120, 260
Zinc ions (Zn^{2+})	180, 350, 700, 1300

The cultures were incubated for 5 or 30 minutes before addition of 100 µl 5mM $CaCl_2$. Luminescence was measured for 5 minutes using a plate luminometer. Luminescence data was manually converted from relative light units to cytosolic free calcium ion concentration, $[Ca^{2+}]_c$. This was then plotted against time and parameters of this trace were analysed. Parameters assessed were as follows:

Rise Time, the time from addition of $CaCl_2$ to the moment when maximum $[Ca^{2+}]_c$ was achieved;
Amplitude, the maximum $[Ca^{2+}]_c$ reached during the experiment;
Full Width Half Maximum, the width of the transient at the point where the amplitude equals half of the maximum amplitude of the transient;
and Final Resting $[Ca^{2+}]_c$ Level, the resting $[Ca^{2+}]_c$ at the end of the experiment.

<u>Effects of further toxicants on $[Ca^{2+}]_c$ traces</u>

Cultures of *Aspergillus awamori* as described above were used to test the effects of further toxicants. The concentrations of toxicants tested were made up

as follows in water, where the concentrations tested were based on Dutch target and intervention values for toxicants and Kelly Guidelines for the classification of contaminated soils:

TOXICANT	CONCENTRATION (mg/l)
Pentochlorophenol, PCP	0.01, 0.1, 1, 5, 10
Sodium dodecyl sulphate, SDS	1, 10, 50, 100, 500
Toluene	1, 25
3,5-DCP	10
Zn^{2+}	700
Cr^{6+}	15

In the first set-up (S1), 100 µl of each toxicant concentration or of the control (VS medium) were added to the cultures through built-in injectors and luminescence monitored for 5 minutes. In a second set of experiments (S2), cultures were pre-incubated with the toxicant or control for 5 minutes before addition of 5mM $CaCl_2$ in a total volume of 25 µl distilled water. Luminescence was monitored for 5 minutes following addition of $CaCl_2$. Luminescence data was converted from relative light units to $[Ca^{2+}]_c$ values as described above. The following parameters were assessed in S1:

Lag Time, the time from addition of $CaCl_2$ to the time when $[Ca^{2+}]_c$ began to rise;

Rise Time;

Amplitude;

Full Width Half Maximum;

Percentage Increase in Final $[Ca^{2+}]_c$ Resting Level, where the control value was taken to be 100%; Percentage Increase in Recovery Time, where the control value was taken to be 100%; and Number of $[Ca^{2+}]_c$ Increases, the number of $[Ca^{2+}]_c$ transients.

In S2, the Percentage Increase in pre-Stimulating $[Ca^{2+}]_c$ Resting Level, where the control value was taken to be 100%, was assessed in addition to all of the parameters tested in S1.

<u>Effects of mixtures containing different proportions of toxicants on $[Ca^{2+}]_c$ traces</u>

The experiments described when examining the effects of further toxicants were repeated for different mixtures of toxicants. The following mixtures were made up in water for testing:

6 mg/l 3,5-DCP + 12 mg/l Cr^{6+}
30 mg/l Cr^{6+} + 350 mg/l Zn^{2+}
10 mg/l 3,5-DCP + 350 mg/l Zn^{2+}
6 mg/l 3,5-DCP + 12 mg/l Cr^{6+} + 350 mg/l Zn^{2+}

Mixture 1:	20 mg/l Cadmium
	100 mg/l Copper
	50 mg/l Chromium
	250 mg/l Zinc
	500 mg/l SDS
Mixture 2:	20 mg/l Cadmium
	100 mg/l Copper
	50 mg/l Chromium

250 mg/l Zinc

These experiments demonstrate a novel finding that each toxicant results in a different and characteristic $[Ca^{2+}]_c$ transient. Additionally each concentration of toxicant produces a unique $[Ca^{2+}]_c$ transient. From these characteristic fingerprint responses a profile of data can be built up and used to create a bank of data for each toxicant. Results from testing samples can be compared with this data bank and the presence of a particular toxicant can thus be determined. Furthermore, details such as the mode of action of the toxicant, and the amount of toxicant present can be deduced from a comparison with the bank of pre-gathered data.

Although the invention has been particularly shown and described with reference to particular examples, it will be understood by those skilled in the art that various changes in the form and details may be made therein without departing from the scope of the present invention.

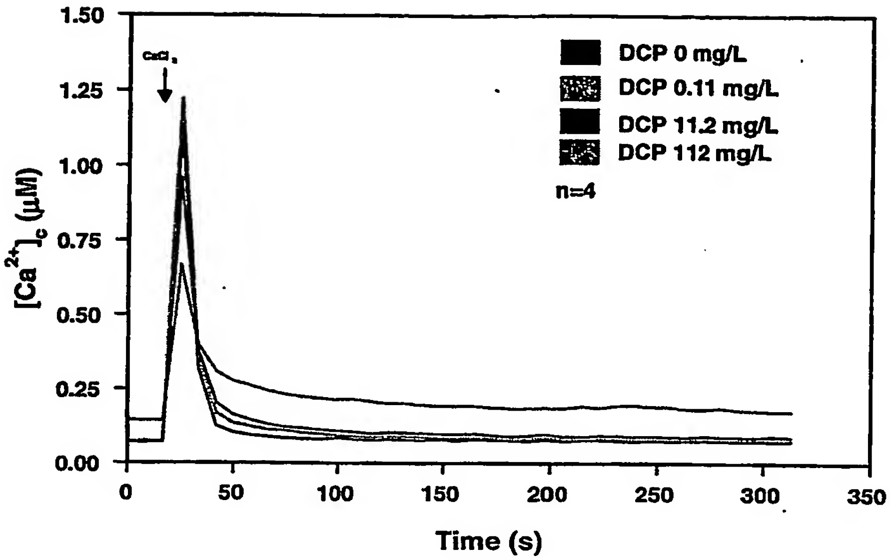


Figure 1

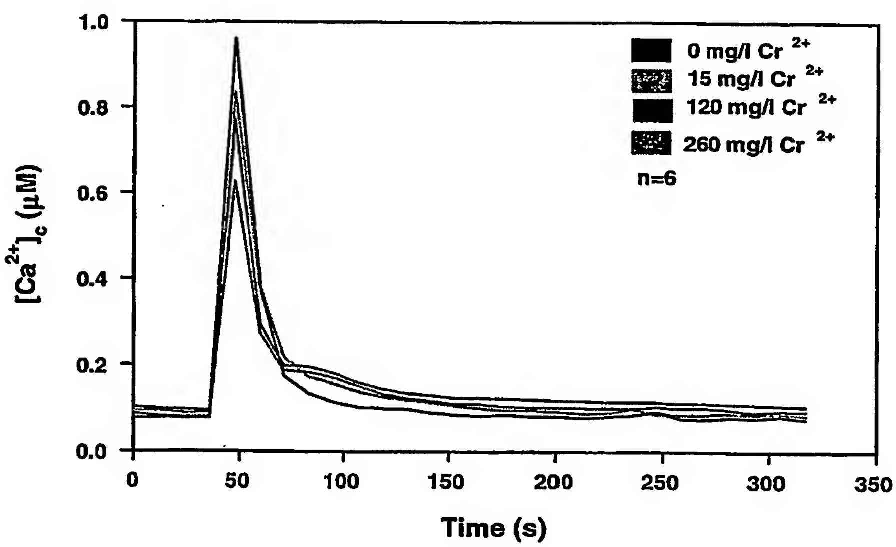


Figure 2

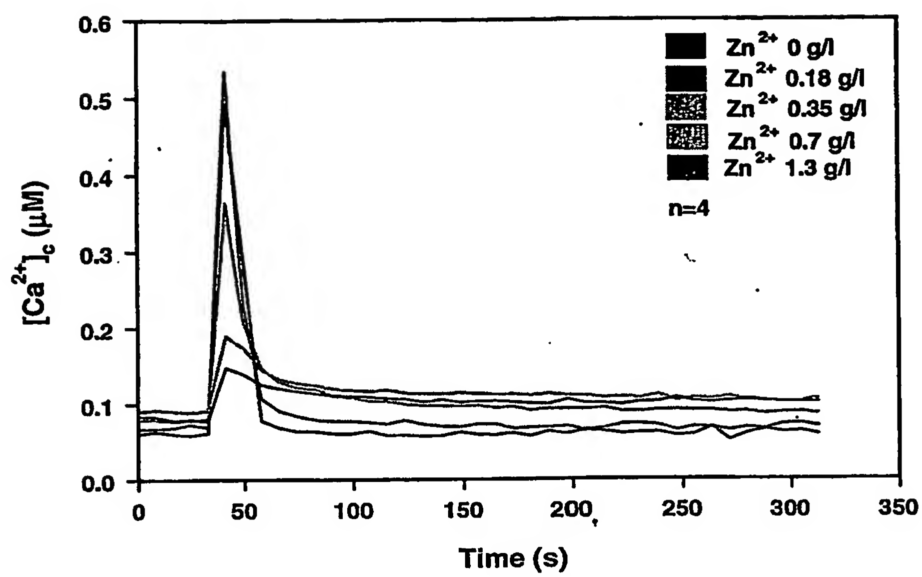
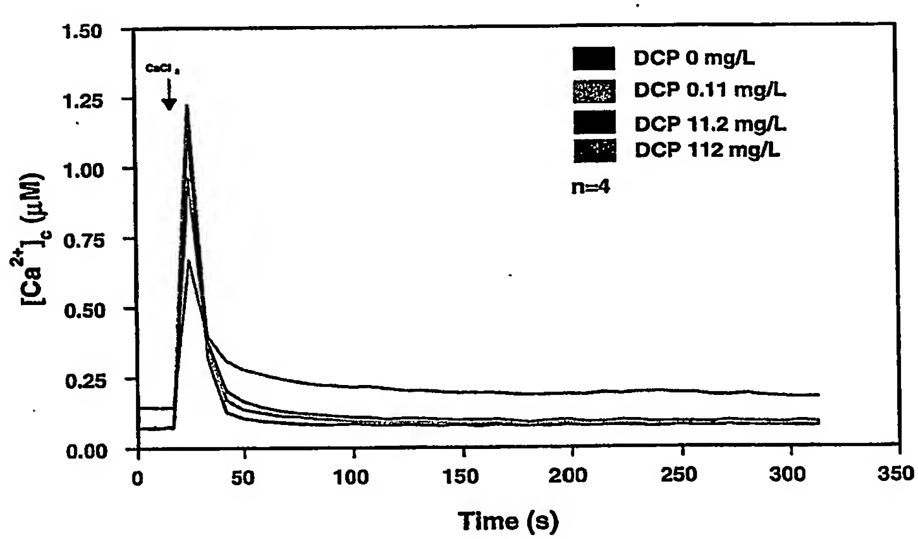


Figure 3



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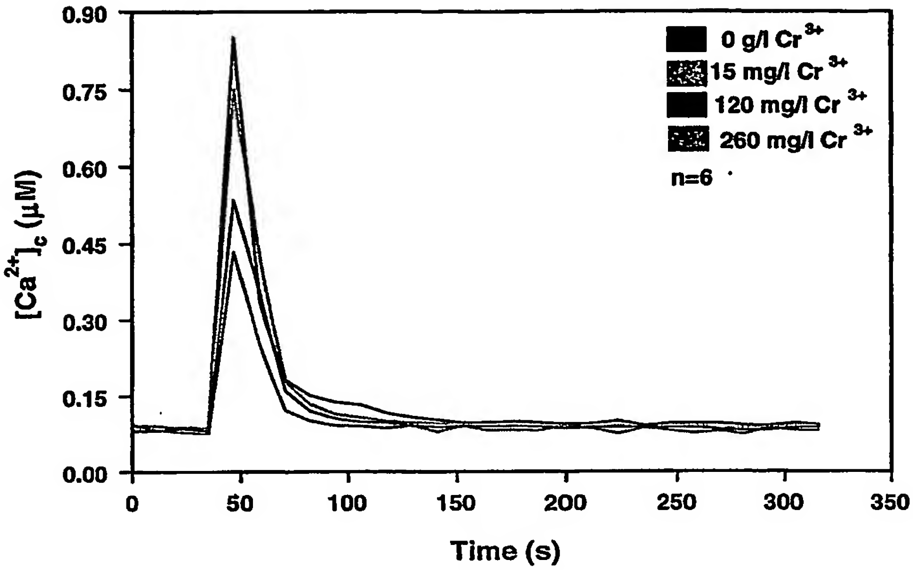

Figure 5

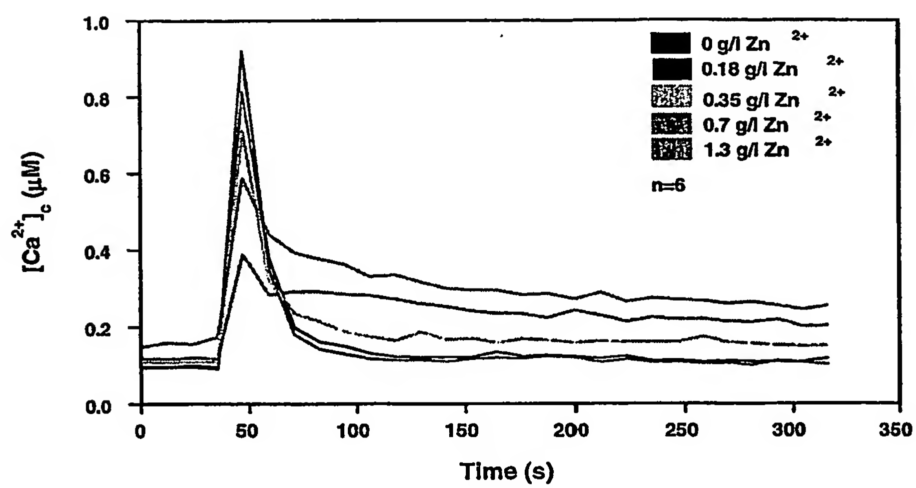

Figure 6

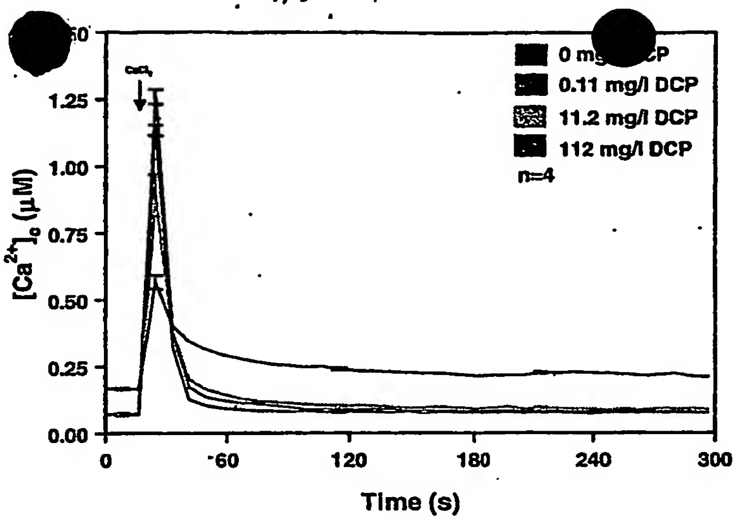

Figure 7

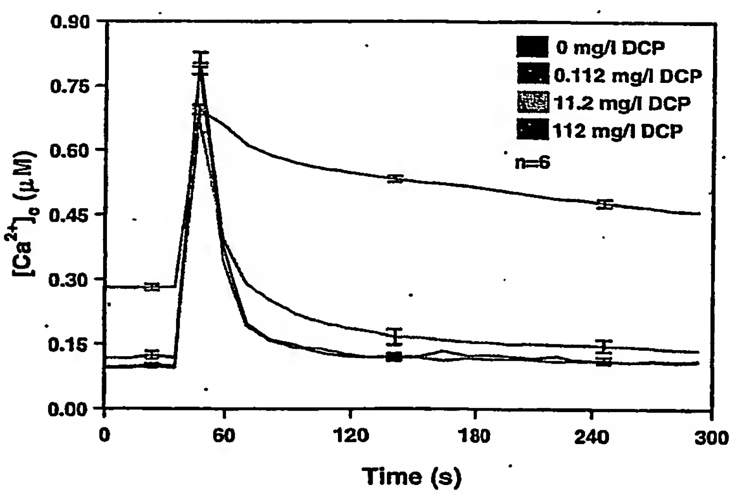

Figure 8

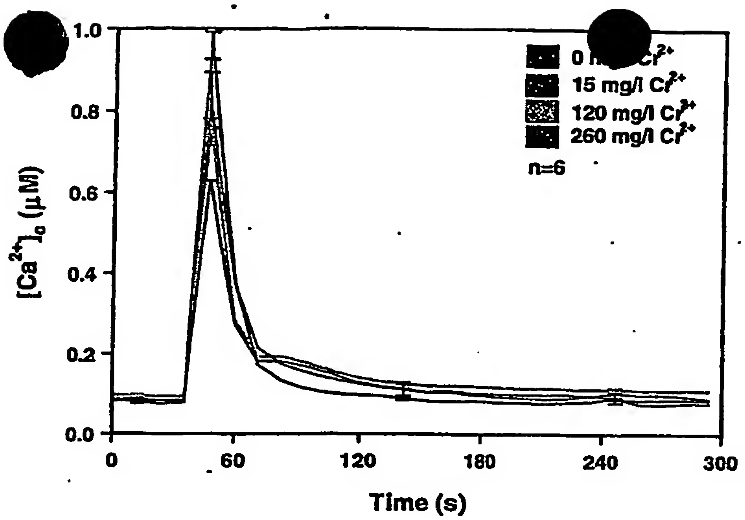

Figure 9

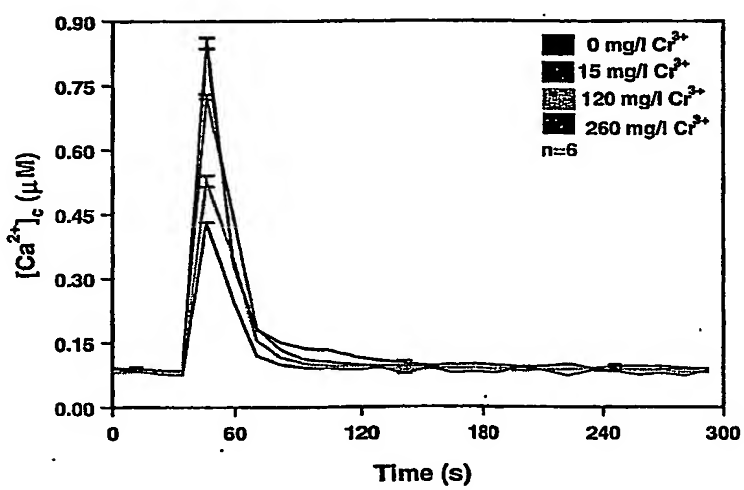

Figure 10

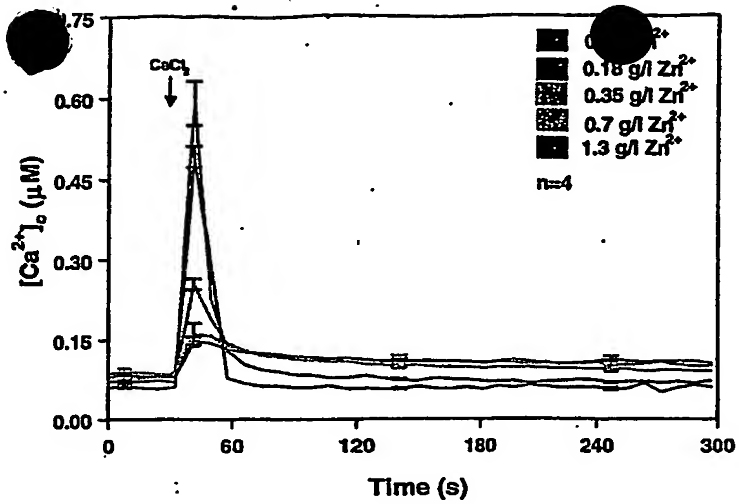

Figure 11

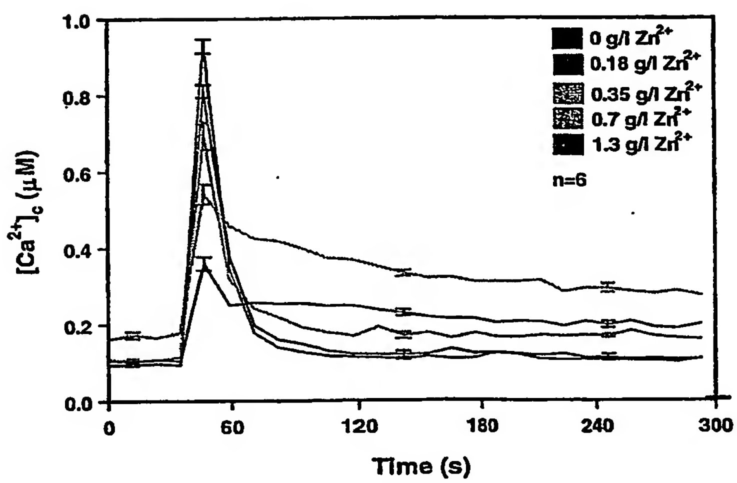

Figure 12

Chemical	Values of interest (mg/l)	LT		RT		A		FWHM		%IpreSRL	%IFRL		%IRT		Number of increases	
		S1	S2	S1	S2	S1	S2	S1	S2	S2	S1	S2	S1	S2	S1	S2
	0.01	0	0	1	1	75±23	100±5	↑	-	97±9	115±6	104±6	111±4	102±3	1	1
	0.1	0	0	1	1	74±22	91±5	↑	-	104±9	118±5	104±11	122±9	99±3	1	1
PCP	1	0	0	1	1	72±14	80±10	↑	-	109±5	204±13	107±11	195±8	109±6	1	1
	5	0	0	12.6	1	199±26	89±14	↑	↑	120±3	209±7	205±9	274±4	218±7	1	1
	10	0	0	12.6	12.6	417±73	175±27	↑	↑	131±9	305±17	253±7	373±22	308±4	1	1
	1	0	0	1	1	111±48	102±8	-	-	125±34	116±5	106±7	114±6	109±9	1	1
	10	0	0	12.6	1	246±19	118±4	↑	↑	136±13	162±7	120±13	243±14	154±10	1	1
SDS	50	0	0	12.6	1	295±33	115±12	↑	↑	323±19	237±9	222±16	328±6	287±10	1	1
	100	0	0	12.6	1	293±19	116±3	↑	↑	359±24	405±15	256±14	367±11	286±12	1	1
	500	0	n.a.	12.6	n.a.	998±35	n.a.	↑	n.a.	n.a.	561±12	n.a.	565±12	n.a.	1	n.a.
Toluene	1	n.a.	0	n.a.	1	n.a.	74±13	n.a.	-	129±9	n.a.	113±3	n.a.	115±2	n.a.	1

Note: LT= lag time
RT=rise time
A=changes in A (%)
FWHM=full width half maximum

%IpreSRL= % increase in pre-stimulation resting level
%IFRL=% increase in final resting level
%IRT=% increase in recovery time
S1=Stage 1
S2=Stage 2

Figure 13

Chemical	Values of interest (mg/l)	LT		RT		A		FWHM		%IpreSRL	%IFRL		%IRT		Number of increases	
		S1	S2	S1	S2	S1	S2	S1	S2	S2	S1	S2	S1	S2	S1	S2
3,5 DCP	10	0	0	12.6	1	40±2	79±25	↑	↑	102±3	134±10	172±21	153±13	207±23	1	1
PCP	10	0	0	12.6	12.6	417±73	175±27	↑	↑	131±9	305±17	253±7	373±22	308±4	1	1
Zn^{2+}	700	0	0	12.6	1	42±5	74±1	↑	↑	142±18	225±8	263±14	258±5	225±12	1	1
Cr^{6+}	15	0	0	1	1	41±5	84±23	-	-	102±12	104±5	110±11	103±2	120±28	1	1
Toluene	25 (1)	0	0	n.a.	1	n.a.	73±13	n.a.	↑	121±6	n.a.	113±3	n.a.	115±2	1	1
SDS	500	0	0	12.6	1	998±35	116±3	↑	↑	359±24	561±12	256±14	565±12	286±12	1	1

Note: LT= lag time
RT=rise time
A=changes in A (%)
FWHM=full width half maximum

%IpreSRL= % increase in pre-stimulation resting level
%IFRL=% increase in final resting level
%IRT=% increase in recovery time
S1=Stage 1
S2=Stage 2

Figure 14

Chemical	Values of interest (mg/l)	LT		RT		A		FWHM		%IpreSRL	%IFRL		%IRT		Number of increases	
		S1	S2	S1	S2	S1	S2	S1	S2	S2	S1	S2	S1	S2	S1	S2
3,5 DCP	10	0	0	12.6	1	40±2	79±25	↑	↑	102±3	134±10	172±21	153±13	207±23	1	1
Cr^{6+}	15	0	0	1	1	41±5	84±23	-	-	102±12	104±5	110±11	103±2	120±28	1	1
Zn^{2+}	700	0	0	12.6	1	42±5	74±1	↑	↑	142±18	225±8	263±14	258±5	225±12	1	1
SDS	500	0	0	12.6	1	998±35	116±3	↑	↑	359±24	561±12	256±14	565±12	286±12	1	1
3,5-DCP + Cr^{6+}	6+12	0	0	12.6	1	33±4	88±13	↑	-	96±26	117±4	120±14	128±5	119±10	1	1
Cr^{6+} Zn^{2+}	30+350	*0*	0	*1*	1	*23±6*	76±4	↑	-	100±5	*143±18*	150±12	*158±26*	154±6	*1*	2
3,5DCP +Zn^{2+}	10+350	*0*	0	*12.6*	1	*65±5*	86±2	↑	-	103±9	*294±18*	153±16	*311±14*	208±11	*1*	2
3,5-DCP + Cr^{6+} Zn^{2+}	6+12+350	*0*	0	*12.6*	1	*25±2*	79±5	↑	-	102±8	*164±4*	150±9	*160±5*	195±8	*1*	2
Mixture 1	See M&M	0	0	12.6	12.6	466±13	128±6	↑	↑	262±13	402±17	501±38	446±17	477±28	2	2
Mixture 2	See M&M	0	0	1	1	116±8	69±14	↑	-	148±16	177±44	132±27	170±28	120±7	1	1

Italics represents data obtained with very high concentrations of toxicants: Zn^{2+}=700 mg/l; Cr^{6+}=120 mg/l; 3,5 DCP=49 mg/l

Figure 15